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**A STUDY ON THE PHOSPHORUS DISTRIBUTION IN RAT STRIATED MUSCLE
AS INFLUENCED BY AGE, DIET,
AND IRRADIATED ERGOSTEROL**

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY
AND PHARMACOLOGY

By
VERSA VIOLA COLE

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A STUDY ON THE PHOSPHORUS DISTRIBUTION IN RAT STRIATED MUSCLE AS INFLUENCED BY AGE, DIET, AND IRRADIATED ERGOSTEROL

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The object of this study was to determine the phosphorus distribution in the gastrocnemius muscle of the albino rat as influenced by: (a) our normal stock diet, (b) age, (c) McCollum's Diet 3143, (d) McCollum's Diet 3143 plus irradiated ergosterol, and (e) length of time on the McCollum rachitic diet. We studied the effect of these factors on five phosphorus fractions: acid-insoluble, acid-soluble, labile plus inorganic, labile, and total phosphorus. The total phosphorus is the sum of acid-insoluble and acid-soluble phosphorus. The acid-insoluble phosphorus is the form which is not extracted by grinding with 5 per cent trichloroacetic acid and sand, followed by washing with trichloroacetic acid, and includes, of course, the phosphorus in protein and lipid combinations. The acid-soluble phosphorus represents the total phosphorus in the trichloroacetic acid extract from the muscle and includes in addition to the labile and inorganic phosphorus, lactacidogen phosphorus, pyrophosphate, adenylic acid phosphorus, and other organic phosphorus combinations. The labile plus inorganic phosphorus value as used by us was called inorganic phosphorus up to the work of Eggleton and Eggleton (1) and Fiske and Subbarow (2). The labile phosphorus is probably all phosphocreatine, as shown by the work of Fiske and Subbarow (3).

For the weaning group we used rats from two groups taken 3 weeks apart; one group from three litters and the other group from one litter. The number of experiments on each group of the same

age of the rachitic rats, the dosage of irradiated ergosterol in terms of the therapeutic dose, the number of animals both experimental and control, and the degree of rickets in the controls are given in Table I. Only one experiment was carried out on each group of animals of the same age on the stock diet. The average

TABLE I
Summary Giving Dosages and Degrees of Rickets

No. of experiments	Age	Length of experiment	Dosage of ergosterol times therapeutic dose	No. of rats		Rickets in controls
				Experimental	Control	
	<i>days</i>	<i>days</i>				
1	36	15	4,300	1	1	R
2	39	16	4,300	3	2	2+, 3+
3	35	13	2,800	1	1	2+
1	48	22	4,300	2	2	4+
2	49	28	4,300	2	2	4+
3	48	24	4,300	3	2	2+, 3+
4	48, 47	24	4,300	2	2	R, 3+
1	66	45	8,600	3	3	S R
2	73	48	8,600	5	5	2R, 2+, 3+
3	71	49	8,600	5	5	3S R, +, 3+
1*	87	66	4,300	1	1	4+
2*	88	67	4,300	3	3	4+
3	92-93	70	2,800	5	5	2+, 3+
4	89-92	67	30 days, 2,800 37 " 20,000	6	6	2SR, 2+ 3+
1	120±7	97	8,600	3	3	4+
1	190	169	10,000	4	4	4+

R = rickets; S R = severe rickets.

* Used only for the average of rickets with and without ergosterol.

minimum and maximum values for each type of phosphorus for each group of animals are given in Tables II and III.

Methods

The stock rats were fed our stock diet consisting of milk, sunflower seed, and a mixture of 25 per cent powdered milk and 75

TABLE II

Effect of Age on Distribution of Phosphorus in Mg. per 100 Gm. of Muscle

Age	Diet	Length of experiment	Labile + inorganic P				Labile P			
			No. of rats	Minimum	Maximum	Average	No. of rats	Minimum	Maximum	Average
<i>days</i>		<i>days</i>								
21	Just weaned		10	72	101	84	8	41	58	49
35-39	R	13-16	4	106	109	107	3	63	76	71
35-39	R + E	13-16	5	102	106	104	5	64	81	73
35-39	R (all)	13-16	9	102	109	105	8	63	81	73
35	S	14	4	103	116	107	3	64	82	77
47-49	R	22-28	8	85	130	110	3	58	79	70
47-49	R + E	22-28	9	103	129	110	6	57	82	73
47-49	R (all)	22-28	17	85	130	110	9	57	82	72
66-73	R	45-49	14	89	149	117				
66-73	R + E	45-49	14	100	140	119				
66-73	R (all)	45-49	28	89	149	118				
75	S	54	4	108	117	113	3	62	83	75
87-95	R	66-70	12	125	159	132	8	59	86	72
87-95	R + E	66-70	12	124	159	134	7	68	87	74
87-95	R (all)	66-70	31	133	159	133	15	59	87	73
93	S	71	5	107	122	116	4	62	71	66
120±7	R	97	3	108	120	116	3	82	85	83
120±7	R + E	97	3	112	123	118	3	60	79	71
120±7	R (all)	97	6	108	123	117	6	60	85	77
120±14	S	99±14	4	90	105	99	4	59	89	75
190	R	159	3	111	118	116	4	66	88	76
190	R + E	159	3	106	126	116	2	79	90	84
190	R (all)	159	6	106	126	116	6	66	90	79
168	S	147	4	92	97	95	2	70	74	72

S = stock diet; R = rachitic diet; R + E = rachitic diet plus irradiated ergosterol; R (all) = rachitic diet with and without ergosterol.

per cent ground yellow corn daily, and in addition, twice a week, a bread and meat mixture and lettuce or carrots. The experimental diet, with and without irradiated ergosterol, consisted of the

TABLE III

Effect of Age on Distribution of Phosphorus in Mg. per 100 Gm. of Muscle

Age	Diet	Length of experiment	Acid-insoluble P				Acid-soluble P				Acid-insoluble plus acid-soluble P			
			No. of rats	Minimum	Maximum	Average	No. of rats	Minimum	Maximum	Average	No. of rats	Minimum	Maximum	Average
<i>days</i>		<i>days</i>												
21	Just weaned		9	81	93	89	9	125	159	140	8	214	239	225
35-39	R	13-16	3	46	68	54	4	142	171	159	3	187	238	210
35-39	R + E	13-16	4	45	52	48	5	150	172	157	4	198	216	206
35-39	R (all)	13-16	7	45	68	51	9	142	172	157	7	187	238	208
35	S	14	4	71	79	76	4	169	189	179	4	247	266	255
47-49	R	22-28	5	55	68	61	8	150	178	162	5	205	237	222
47-49	R + E	22-28	6	42	61	52	9	153	186	163	8	194	244	213
47-49	R (all)	22-28	13	36	68	52	17	150	186	162	13	194	244	217
66-73	R	45-49	9	21	39	29	13	144	180	163	9	170	213	194
66-73	R + E	45-49	10	21	35	26	15	153	183	167	10	182	207	195
66-73	R (all)	45-49	19	21	39	28	28	144	183	165	19	170	213	195
75	S	54	3	51	64	56	4	168	186	177	3	210	243	227
87-95	R	66-70	11	27	45	34	12	162	205	180	10	193	243	216
87-95	R + E	66-70	11	29	41	32	12	171	210	182	10	200	240	214
87-95	R (all)	66-70	29	26	45	33	30	158	210	179	25	193	243	213
93	S	71	4	50	53	52	5	161	175	166	4	213	227	220
120±7	R	97	3	45	53	49	3	152	184	169	2	201	225	213
120±7	R + E	97	3	46	50	49	3	170	194	179	2	225	239	231
120±7	R (all)	97	6	45	53	49	6	152	184	174	4	201	239	222
120±14	S	99±14	3	51	56	53	4	161	180	171	3	213	233	226
190	R	159	3	48	52	50	4	169	183	177	3	217	233	225
190	R + E	159	3	50	54	52	4	166	180	173	2	216	233	224
190	R (all)	159	6	48	54	51	8	166	183	175	5	216	233	225
168	S	147	4	49	56	52	4	155	164	160	4	212	213	212

S = stock diet; R = rachitic diet; R + E = rachitic diet plus irradiated ergosterol; R (all) = rachitic diet with and without ergosterol.

McCollum Diet 3143.¹ The ergosterol-treated animals were given daily, by medicine dropper, the peanut oil containing the irradiated ergosterol, in doses representing 2800 to 20,000 rat units. We used both males and females on the rachitic diet with a predominance of males. All the normal animals older than the weaning age were males.

The gastrocnemius muscles were obtained from the rat under barbital-ether anesthesia. The gastrocnemius was dissected free from the surrounding tissues, a thread passed under the tendon of Achilles and tied tightly, and the tendon cut distal to the thread. The other end of the thread was tied to a staple in the end of a 15 inch stick. If the two muscles were to be analyzed together they were both dissected out one after the other and tied to two separate sticks. If they were to be analyzed separately, as in a few of our earlier experiments, one muscle was dissected out, frozen, and weighed, etc. before the other was dissected out.

The rat was carried into the freezing room at -4° . With one clip of the scissors each gastrocnemius was removed and dropped at once into liquid air. The muscles were allowed to remain in liquid air from 2 to 10 minutes, and then removed, cut from the thread, weighed to the nearest mg., placed in a chilled porcelain mortar, broken up, and ground with sand in the freezing room. Next 10 or 20 cc., depending upon the weight of the muscle, of ice-cold 5 per cent trichloroacetic acid were added while grinding. After thorough mixing, the extract was filtered through quantitative filter paper in a cold funnel resting in a 10 cc. graduated centrifuge tube specially prepared for this purpose. 2 cc. of the filtrate were collected and the funnel was transferred to another centrifuge tube and 2 cc. more of the filtrate were collected for a duplicate determination on labile phosphorus. A drop of phenolphthalein was added to each tube and 2 per cent sodium hydroxide was added, while stirring, to the end-point of phenolphthalein. Another 1 cc. filtrate was collected for total acid-soluble phosphorus.

The tubes were then removed from the freezing room and the inorganic phosphorus precipitated with the calcium mixture of Fiske and Subbarow (3). Our determinations were made accord-

¹ McCollum, E. V., [Simmonds, N., Shipley, P. G., and Park, E. A., *J. Biol. Chem.*, **47**, 507 (1921).

ing to Fiske and Subbarow's method except that we precipitated the excess calcium from the filtrate with the sulfuric acid necessary to develop color in their colorimetric method, removed the precipitate by centrifuging, and then used a neutral molybdate solution. The volume was made up to 25 cc. for comparison in the colorimeter. 2 cc. of the trichloroacetic acid filtrate were used for the determination of labile plus inorganic phosphorus and 1 cc. for the determination of total acid-soluble phosphorus, the volume being made up to 25 cc. in each case. The acid-insoluble phosphorus determination was run on the trichloroacetic acid precipitate with the filter paper and the sand, after washing in the cold with a 5 per cent trichloroacetic acid solution until free from inorganic phosphate. The final volume for the colorimetric determination was 50 cc. or 100 cc. depending upon the weight of the muscle.

Ashing for the total acid-soluble and acid-insoluble fractions was carried out with either sulfuric acid and nitric acid or sulfuric acid and phosphorus-free hydrogen peroxide. The acid-insoluble phosphorus determinations were never performed in duplicate because only one sample was available. The determinations on the filtrate were run in duplicate except when the two gastrocnemii were analyzed separately and on the rats just weaned. When the two gastrocnemii were analyzed separately, the determinations from the two muscles were taken as duplicates. There was not enough material from the rats just weaned to make duplicate determinations on anything except labile phosphorus. All phosphorus determinations were carried out by the colorimetric method of Fiske and Subbarow (4). The calculations for the phosphorus fractions in the acid-soluble portion were based on the assumption that the total volume of the solution was equal to the trichloroacetic acid added plus three-fourths the weight of the muscle.

DISCUSSION

The labile phosphorus varied considerably among rats of the same group, and it did not seem to be any more variable among rats of different ages than among those of the same age if we except the weaning value. The labile phosphorus within the limits of error of our methods was not influenced by the rachitic diet either

with or without ergosterol. It is the only fraction which was the same throughout for rats on both normal and rachitic diets. As a basis for an explanation of the low weaning value as compared with the higher values at all later ages, natural activity of the animal suggests itself in view of the very striking creatinephosphate increase which Ferdmann and Feinschmidt (5) found by training muscles. At weaning, the animals had not used their hind legs much nor for very long, and the creatinephosphate was low; however, within 2 more weeks the animals became very active and the creatinephosphate probably reached the maximum and constant values.

As for the labile plus inorganic phosphorus, there were no differences between the curves for the animals on the rachitic diet only and those on the rachitic diet with irradiated ergosterol. The values for labile plus inorganic phosphorus for the normal animals after 70 days of age, were below those for animals of the same age on the rachitic diet, which is the reverse of the observations of Hentschel and Zoeller (6). They obtained lower values for this fraction in rats kept for 2 to 8 weeks on the rachitic diet; otherwise their values ran about the same as ours. Thus, their highest values came at about 12 or 13 weeks of age, which was the oldest group they used, and our highest values came at about the same age. They do not state what their stock diet was, so that we cannot tell whether the difference was due to a variance in stock diets or to some other cause. Uselli (7), working on a similar fraction in ox striated muscle, found a continuous increase from the 3 cm. fetus to the adult ox, calculated on the basis of moist weight, and scarcely any change calculated on the basis of dry weight.

The acid-soluble phosphorus was not affected by irradiated ergosterol. In the 2 weeks following weaning, there was a rapid rise in both rachitic and normal groups, but the rise in the normal was about twice as great as the rise in the rachitic groups. The highest level for the stock animals was reached by the end of 2 weeks after weaning, while the animals on the rachitic diet did not reach this level until they were 3 months of age and by that time the normal rats showed a falling off and their final level was below that of the rachitic rats.

Probably our most interesting fraction from the standpoint of

changes affected by age is the acid-insoluble phosphorus. This fraction had its highest value at the weaning age, which is the age for which Sinclair (8) showed the highest phosphatide content of the whole rat. The value decreased fairly rapidly in both groups of animals, reaching its lowest values at $2\frac{1}{2}$ to 3 months of age, but a much lower level in the rachitic than in the normal animals. By 4 months of age both groups showed the same values as those reached by the stock rats at 3 months of age. In the rats up to 3 months there seemed to be a slight difference in this fraction between those receiving irradiated ergosterol and those not. The difference produced by the ergosterol, if there was any, made the values differ still more from the normal. These results suggest that the irradiated ergosterol simply hastened the transfer of muscle phosphorus to bone formation at the expense of this type of muscle phosphorus. However, the quantitative handling of this fraction was so difficult that the differences may not be significant.

No trend can be shown indicating any difference in the total phosphorus of the muscle of the rats receiving irradiated ergosterol and those not receiving it. Since our total phosphorus is the sum of the two preceding fractions, it is obvious that the stock animals would show a rise in total phosphorus immediately after weaning, while the rats on the rachitic diet would show a decrease. A difference in the same direction between the two groups of rats was maintained up to 3 or 4 months of age and after that it appeared to be in the opposite direction. Our values were lower than those of Hentschel and Zoeller (6), which did not seem to show any significant differences between the normals and the rats on the rachitic diet.

Only one of the phosphorus values maintained a difference in the same direction throughout the whole experiment between the animals on the stock diet and those on the rachitic diet, and that is the inorganic plus labile phosphorus. The differences exhibited in the other fractions in the early part of the feeding experiments had disappeared before the age of 4 months or had actually gone in the opposite direction. These differences are what we should expect if the variety in the two diets lay in the availability of phosphorus to the rat. We have no direct proof, however, that this was the cause because the stock diet varied from the rachitic

diet in other factors than phosphorus content and calcium to phosphorus ratio. We can say, however, that we believe with confidence that the difference between the effects of rachitic and stock diets on muscle phosphorus was not in the content of vitamin D.

CONCLUSIONS

A comparative study on the distribution of various forms of phosphorus in the gastrocnemius muscles of the albino rat on the stock diet and on the rachitic diet with and without viosterol leads to the following conclusions:

1. A rachitic diet causes a marked fall in the total, acid-insoluble, and acid-soluble phosphorus content.

2. The total phosphorus on the rachitic diet following weaning falls to a very low value in 50 days and then rises to a constant normal adult level; while on the stock diet there is an initial rise and then a gradual fall to the adult level.

3. The acid-insoluble phosphorus content is highest at weaning and then falls more rapidly and to a lower level in the rachitic animals, but in 90 to 100 days rises to the normal adult level.

4. The acid-soluble phosphorus value is lowest at weaning and rises more rapidly on the stock diet than on the rachitic diet. After 60 days the values are essentially the same on the two diets.

5. The labile plus inorganic phosphorus content is lower on the stock diet than on the rachitic diet after the first 5 weeks following weaning.

6. The labile phosphorus content rises abruptly in 2 weeks following weaning to normal adult values, even on the rachitic diet.

7. Irradiated ergosterol in doses from 2800 to 20,000 times the therapeutic dose did not alter the values on the rachitic diet.

The irradiated ergosterol used in these experiments was very kindly furnished by Mead Johnson and Company.

We wish to express our thanks to Mr. J. T. Hauch for allowing us to use some of his animals and to Miss Martha Johnson for her assistance in the chemical work.

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